Genotyping of unusual phenotypes of epidermolysis bullosa

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Chapter 3

Revertant mosaicism in epidermolysis bullosa due to different second-site mutations in *LAMB3*

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Abstract

Revertant mosaicism due to *in vivo* reversion of an inherited mutation has been described in the genetic disease epidermolysis bullosa (EB) for the genes *KRT14* and *COL17A1*. Here, we demonstrate the presence of several different second-site mutations, all correcting the germ-line *LAMB3* mutation 628G→A, in two unrelated mosaic nH-JEB patients. Both patients had a severe reduction of laminin-332 expression in their affected skin. Remarkably, patient EB 078-01 (628G→A/R635X) obtained clinically healthy skin on his lower leg with normal expression of laminin-332 on previously affected skin. In the other proband, EB 029-01 (628G→A/628G→A), the revertant patches were located at his upper arm and shoulder. DNA-analysis showed in revertant keratinocytes of distinct biopsy specimens different second-site mutations: 596G→C, 628+42G→A and 629-1G→A, implying that there is not a single preferred mechanism for the correction of a specific mutation. All three compensatory mutations affected splicing, either by strengthening the natural 5' splice site of intron 7 in case of 596G→C, or by giving rise to aberrant in-frame mRNA transcripts that result in functional protein. Our data offer prospects for EB treatment, since revertant mosaicism seems to occur at a higher frequency than expected, and most importantly, the healthy skin of EB 078-01 expanded over time. This opens the possibility of applying cell therapy in mosaic EB of the *LAMB3* gene using the patient's own naturally corrected keratinocytes.

Figures 1, 2, 4, 7 and 9 of this chapter can be found in the appendix Color figures on page 185.

Introduction

Reverse mutations in germ line or somatic cells bearing an inherited disease-causing mutation can change the phenotype from affected to normal by re-expression of the deficient protein. A reverse mutation can be a true back mutation, which reverts the original mutation to the wild-type codon with subsequent production of the normal wild-type protein [1]. A second possibility is an additional second-site mutation that compensates for the effect of the primary inherited mutation. For instance, a nonsense mutation can be converted into a different amino acid due to such an additional nucleotide change. The phenomenon of revertant mosaicism has been shown in a number of genetic diseases, like tyrosinemia type I [MIM 276700], Bloom syndrome [MIM 210900], Omenn syndrome [MIM 603554], Fanconi anemia [MIM 227650], Wiskott-Aldrich syndrome [MIM 301000], X-linked severe combined immunodeficiency [MIM 300400], adenosine deaminase deficiency [MIM 102700] and epidermolysis bullosa (EB) (for reviews, see [2,3]). EB is a clinically heterogeneous group of heritable blistering disorders leading to fragility of the skin and mucous membranes. On basis of the level of dermal-epidermal separation EB is traditionally divided into three categories; EB simplex, junctional EB and dystrophic EB [4]. Mutations in the genes encoding keratins 5 (*KRT5*) and 14 (*KRT14*), and plectin (*PLEC1*) result in EB simplex that has the blister cleavage plane through the basal cells. In junctional EB, which is conventionally caused by mutations in the genes encoding the proteins type XVII collagen (*COL17A1*), integrin $\alpha 6\beta 4$ (*ITGA6* and *ITGB4*) or laminin-332 (LM-332; *LAMA3*, *LAMB3* and *LAMC2*), the separation is found within the lamina lucida of the basement membrane zone (BMZ) [5-8]. In the most severe form - dystrophic EB - due to deficiency of the type VII collagen protein the cleavage is located beneath the lamina densa. The recessively inherited junctional variant can be subdivided into the Herlitz (lethal) and the less severe non-Herlitz variant (non-Herlitz). Mutations causing premature termination codons (PTCs) on both alleles in one of the LM-332 genes result in the Herlitz EB phenotype, whereas a combination of a null mutation with a missense mutation or a splice-site mutation leads to reduced LM-332 expression and the non-Herlitz junctional EB phenotype [9,10]. The latter form, also known as generalized atrophic benign epidermolysis bullosa (GABEB [MIM 226650]), is characterized by generalized skin blistering since birth, dental anomalies, universal alopecia, and nail dystrophy [10,11]. The LM-332 protein, formerly known as laminin-5 [12], is synthesized by the basal epidermal cells and forms the major adhesion ligand for connecting epithelial cells to the underlying BMZ. LM-332 is also highly important for cell migration

[13]. In invading epithelial cells the protein is highly overexpressed [14]. Moreover, migration, *in vitro* invasion, and *in vivo* tumorigenicity were all enhanced in an oral squamous cancer cell line with lack of γ2 chain expression [15]. LM-332 consists of three subunit polypeptides, the α3, β3, and γ2 chains, encoded for by the distinct genes *LAMA3*, *LAMB3*, and *LAMC2* respectively [16]. The *LAMB3* gene, located on chromosome 1 (1q32), contains 23 exons giving rise to a cDNA of 3,516 bp that encodes 1,172 amino acids [17]. The long arms of these three chains associate to a coiled-coil rod structure. The heterotrimer is synthesized as a 460-kDa precursor that after secretion into the extracellular matrix undergoes proteolytic processing to a smaller form [18].

Revertant mosaicism has been described in EB for mutations in the genes *COL17A1* and *KRT14* [19-23]. The first reported case involved the reversion of one of the defective *COL17A1* alleles into a wild-type sequence due to a mitotic gene conversion event [19]. In another EB patient the focal expression of type XVII collagen in the skin was the result of a second-site frame-restoring mutation in the *COL17A1* gene [20]. In 2005 we demonstrated that in *COL17A1* revertant mosaic patients multiple correcting *COL17A1* mutations are present in the distinct type XVII collagen-positive skin patches [23]. Based on the pattern of the healthy skin patches from birth on and the fact that the patches did not expand in time, we concluded that these reversion events took place during embryogenesis and that *COL17A1* correction does not provide a selection advantage for revertant keratinocytes.

In the present study we describe two mosaic cases with GABEB caused by germline mutations in the *LAMB3* gene. The skin of the left lower leg of patient EB 078-01 had always been fragile but reverted during life to skin that abolished blistering tendency due to a higher production of LM-332 protein. Mutation analysis of several biopsies disclosed in both patients the presence of different correcting *LAMB3* mutations that all corrected the same inherited 628G→A transition in exon 7.

Materials and Methods

Biopsy sites

For immunofluorescence (IF) microscopy four 4-mm punch biopsies from patient EB 078-01 were taken and snap frozen: one from lesional affected skin from the upper arm, one from nonlesional affected skin from the upper arm, and two from unaffected skin of the left lower leg. For electron microscopy three 2-mm punch biopsies were taken: one from lesional affected skin of the left upper arm, one from nonlesional affected skin of the left upper arm, and one from unaffected skin of the

left lower leg.
The second patient EB 029-01 has been described elsewhere [10,24]. Precisely, patient #9 in the publication of Jonkman et al [10] and patient #2 in the publication of Pulkkinen et al [24]. Two biopsies were studied with IF microscopy: one from nonlesional affected skin from the left upper arm, and one from unaffected skin from the right upper arm.

Immuno-morphological analysis of skin
Details of IF microscopy and electron microscopy have extensively been documented elsewhere [8]. For detection of LM-332 two antibodies were used: K140 specific to the β3 chain (gift from Dr B. Burgeson, Boston, USA) and GB3 recognizing a conformational epitope of the γ2 chain of LM-332 (Abcam Limited, Cambridge, United Kingdom). As marker for junctional EB we used mABs 19-DEJ-1 (gift of dr J.D. Fine, Nashville, USA). Type XVII collagen was stained with 1D1 (gift from Dr K. Owaribe, Nagoya, Japan) and type VII collagen with LH7:2 (gift from Dr I. Leigh, London, United Kingdom). The Alexa488-conjugated goat anti-mouse IgG antibody (Brunschwig Molecular Probes, Amsterdam, the Netherlands) was used as secondary step.

DNA and RNA isolation from skin sections
DNA recovery with laser dissection microscopy (LDM) was performed as described previously [23]. Monoclonal antibody K140 was used to differentiate between keratinocytes with positive (indistinguishable from control) and reduced staining. For DNA isolation about 200 cells were collected in a 0.2 ml reaction tube. Proteinase K digestion was for 60 min at 55ºC followed by proteinase K inactivation at 98ºC for 15 min. The final aliquots were used for PCR.
For RNA isolation, three 10 μm skin sections were transferred to a PCR tube that contained 100 μl of lysis buffer plus 0.7 μl β-mercaptoethanol. Total RNA (15 μl) was then prepared using the Stratagene RNA microprep kit (Stratagene). cDNA synthesis was as described elsewhere [23].

Identification of the mutations in LDM samples
For *LAMB3* (GenBank, accession # U17744-U17760) mutation detection in LDM-isolated DNA we used nested PCR. 1 μl of the first PCR was used for the second PCR. PCR cycling conditions were 5 min at 94ºC, followed by 35 cycles at 94ºC for 45 s, 55ºC for 45 s and 72ºC for 1 min, and a final extension at 72ºC for 7 min. Water, instead of DNA, was used as a negative control. Primer sequences used to amplify the different exons of *LAMB3* and expected product size are listed in table 1. After

PCR, aliquots of 14 μl were examined using 1.5% agarose gel electrophoresis. Also cDNA samples from dissected cells were subjected to nested PCR. All primers were designed in such way that the PCR product contained sequences from multiple exons (table 2). All PCRs were repeated with templates from at least three separate LDM-obtained nucleic-acid isolations. The resulting PCR products were cloned into the pCR4-TOPO vector (Invitrogen). Clones were selected and sequenced.

Accession numbers

GenBank, http://www.ncbi.nlm.nih.gov/GenBank/ (for human *LAMB3* gene [accession numbers U17744-U17760]).

Table 1 PCR amplification of genomic *LAMB3* DNA

Position	PCR[a]	Primer		Product size (bp)
		Forward	Reverse	
Exon 6	1	cactggaggagcctgtctct	cttctcaggagtccgtgtgg	361
	2	tctctgcttgtgtctccacg	cgtgtggctgtgtgaacagt	331
Exon 7: 628G→A	1	ctcaggttccaggtaacaag	ctctccagacctcaaccatc	372
	2	aggtttcttctgaactcggg	gcagggcaagtatcaaatcc	265
Exon 8	1	ggttgaggtctggagaggat	tgtctgtagtctgccctggt	376
	2	ttgaggtctggagaggatgg	tcagcatggccgtgacagaa	262
Exon 13	1	cctggattctacctgtggac	ccacatgcatcagtacagga	330
	2	accaggaatctaggacacag	atgcatcagtacaggacatc	308
Exon 14: 1903C→T	1	taagtcatgtagaaagagcc	gcactgtagaaatgtaagga	583
	2	agaaagagcctcaggtcaga	atgtaaggaaggaccagcat	562
Exon 15	1	attctctgttctgttgcctt	gagaatgcatgaggaatgga	401
	2	ttctgactcagtagcagttg	aatgcatgaggaatggagat	359

[a]The numbers 1 and 2 refer to the first PCR and the second/nested PCR respectively.

Table 2 PCR amplification of *LAMB3* cDNA

Position	PCR[a]	Primer		Product size (bp)
		Forward	Reverse	
Exon 7: 628G→A	1	gagtgtaccagtacctggct	cagacacagacatcgtggac	412
	2	agctggcaggatgttcggtg	gcatggccgtgacagaagca	269
Exon 14: 1903C→T	1	cagtgcaaccagttcacagg	actgctcgtaggctgtgctc	699
	2	agaccggacctatggagacg	ccggaaggctcctgaaggat	589

[a]The numbers 1 and 2 refer to the first PCR and the second/nested PCR respectively.

Results

Patient EB 078-01

The left lower leg reverted later in life

The proband was a 46-year-old male from Belgium who consulted our EB center in 1999 with characteristic GABEB features. His clinically normal parents were unrelated, and his siblings were unaffected. There was no family history of inherited

skin diseases. Since birth the patient displayed generalized trauma-induced skin blistering, with healing without scarring or milia but with some atrophy and hyperpigmentation. The finger and toe nails were dystrophic. He had sparse scalp hair with male pattern boldness, mild alopecia of beard hair, and complete loss of eyebrows, eyelashes and secondary hair. The patient's teeth all had enamel defects and had been extracted at the age of 20. Remarkably, the proband claimed that after an erosive period of 7 years the skin of his left lower leg that had always shown blisters after minor trauma, healed to clinically unaffected skin where no trauma-induced blisters could be evoked (fig. 1). The reversion of the phenotype was after birth and therefore by definition would be classified as late-onset revertant mosaicism [3]. On the other leg (right) he developed multiple spinocellular carcinomas, for which he was treated by amputation in 1998. One year later metastasis to lymph nodes and lung occurred, resulting in death of the patient.

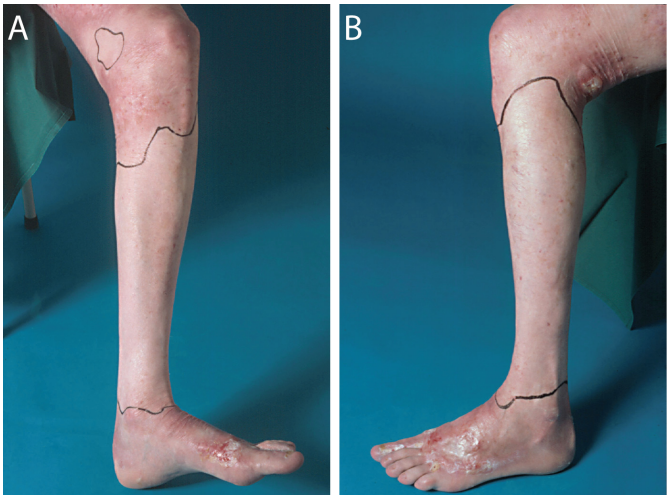

Figure 1 Presence of revertant unaffected skin on the left lower leg of proband EB 078-01 in 1999. The skin of the left lower leg reverted to clinically healthy skin after an erosive period of 7 years *(A medial aspect, B lateral aspect)*, whereas the skin of the right lower leg remained affected. The area of the revertant skin is indicated by a black marker.

Reduced LM-332 expression in affected skin

IF examination of the biopsy of lesional affected skin of the upper arm showed subepidermal blister formation with the cleavage plane low in the lamina lucida, specific for a form of junctional EB; type XVII collagen was exclusively present in the blister roof, whereas type VII collagen was present in the blister base.

IF microscopy of the specimen of nonlesional affected skin of the upper arm showed reduced binding of mABs GB3 and K140 to LM-332 (fig. 2*A* and 2*B*), and

absent binding of 19-DEJ-1 -the marker for junctional EB- along the whole dermo-epidermal junction.
Surprisingly, sections of the lesional affected skin biopsy revealed a small stretch of approximately 25 revertant basal cells that displayed bright LM-332 staining comparable to normal control skin (fig. 2*C*).

Unreduced LM-332 expression in unaffected skin
Interestingly, both biopsies of the unaffected skin of the left lower leg showed positive staining along the BMZ for LM-332 with the same intensity as age matched human control skin. One biopsy displayed a remarkable mosaic pattern consisting of stretches of normal (3+) and reduced (1+) staining (fig. 2*D*), whereas in the other unaffected skin biopsy all basal cells showed bright staining (3+) (data not shown). In line, staining with 19-DEJ-1 was interruptedly positive in the first, and completely normal in the second biopsy.

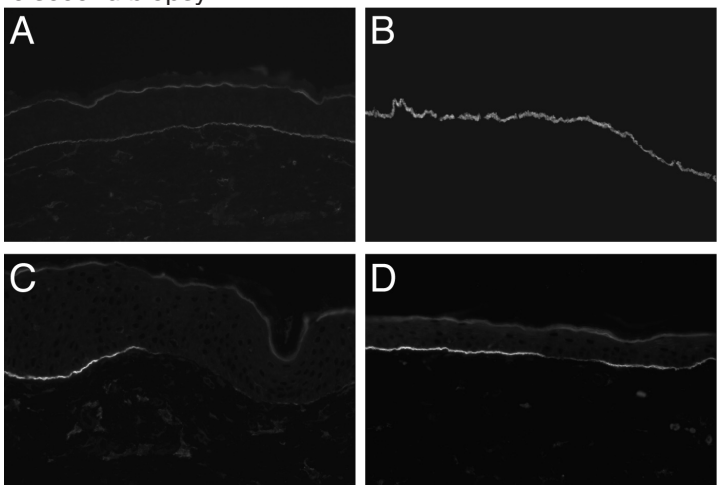

Figure 2 IF microscopy reveals cellular mosaicism in the skin of proband EB 078-01. Staining with monoclonal antibody K140 to the β3 chain of LM-332 was markedly reduced in affected skin of the upper arm (*A*) compared to normal control skin (*B*). To our surprise, a short stretch of approximately 25 LM-332 positive basal cells embedded in LM-332 reduced environment was observed in one of the two clinically affected skin biopsy specimens *(C)*. One biopsy of the revertant skin of the left lower leg showed an interrupted pattern with normal and reduced staining along the epidermal-dermal junction *(D)*, whereas the other biopsy taken from revertant skin revealed bright staining for all basal cells.

Normal hemidesmosomes in revertant skin
Ultrastructural analysis of lesional skin of the left upper arm showed a subepidermal blister with separation at the level of the lamina lucida consistent with a diagnosis of junctional EB. The blister floor was covered by the lamina densa and remainings of tonofilaments were present in the blister cavity. Nonlesional affected skin showed a reduced number of hypoplastic hemidesmosomes and less projection of

tonofilaments than normally (fig. 3*A*). The lamina densa displayed duplications and blind 'off-shoots'.

In unaffected revertant skin the hemidesmosomes were normal in shape (fig. 3*B*). The lamina densa of revertant skin also occasionally showed duplications and blind 'off-shoots'.

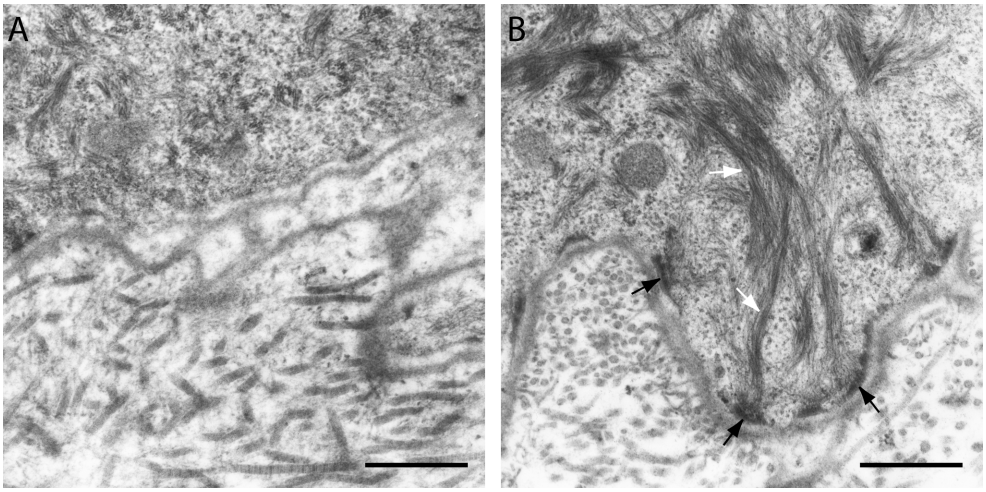

Figure 3 Normal hemidesmosomes in revertant skin. Ultrastructural examination of the affected skin of proband EB 078-01 showed a reduced number of abnormal hemidesmosomes. The intermediate filaments were not connected to the flattened basilar cell periphery *(A)*. The revertant skin of the lower leg reveals intermediate filaments that connect to normal hemidesmosomes in the basilar cell periphery *(B)*. Black arrows indicate hemidesmomes and white arrows the intermediate filaments. Calibration bars represent 500 nm.

Multiple correcting LAMB3 mutations

Since IF microscopy indicated a defect in the LM-332 protein, mutation detection was performed on *LAMA3*, *LAMB3* and *LAMC2* encoding the three chains of LM-332. Sequence analysis revealed compound heterozygosity for two mutations in the *LAMB3* gene in the proband's genomic DNA. The first mutation was a nonsense mutation in exon 14, 1903C→T, leading to the common mutation R635X. On the other allele a transition affecting the last nucleotide of exon 7 (628G→A) was detected, substituting lysine (AAG) for glutamic acid (GAG) at amino acid position 210 (E210K). Since this mutation occurs at position –1 of the 5' donor splice site of intron 7 an effect on mRNA splicing could be expected [24].

To investigate whether the correcting mutations were present in the revertant skin, LDM was performed after immunostaining with K140 to distinguish positive from reduced LM-332 expression. On selected cells DNA analysis was carried out on the mutation-bearing exons 7 and 14, and the neighboring exons 6, 8, 13 and 15. In the biopsy of the lower leg that showed complete reversion, both mutations in

exon 7 and 14 were still present. Fascinatingly, an additional nucleotide change was revealed in intron 7, 628+42G→A, 42 nucleotides away from the exon/intron 7 border (fig. 4*A* and 4*B*). Since the other revertant skin biopsy, with a mosaic reversion pattern, was also taken from the lower leg, we expected here to find the same second-site mutation in intron 7 in the LM-332 bright keratinocytes. Surprisingly, this mutation 628+42G→A was not present, instead another nucleotide change (596G→C) in exon 7 was observed. 596G→C was neither present in DNA isolated from affected skin nor the completely reverted biopsy. This substitution converts a glycine (GGG) residue to an alanine (GCG) residue, and is designated G199A (fig. 4*C*). Cloning and sequencing revealed that this additional transversion (596G→C) was located on the same allele as the inherited splice-site mutation in exon 7. No nucleotide changes in other investigated exons were detected in both revertant biopsy specimens. Furthermore, in fibroblasts taken from unaffected skin the compensatory second-site mutations were absent. Unfortunately, the amount of positive cells in the unaffected skin biopsy was too small -approximately 25 basal cells- to allow successful DNA analysis. Only one single sample could be isolated, whereas we normally perform our sequence analysis on at least three separately obtained samples.

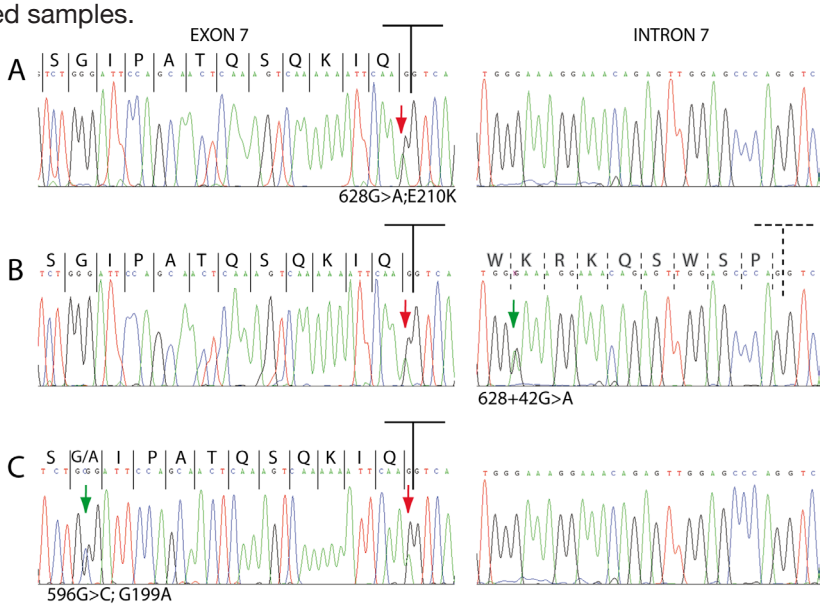

Figure 4 Identification of the different correcting *LAMB3* mutations. The G→A nucleotide change at position -1 of the 5' splice site of intron 7 is present in the keratinocytes of patient EB 078-01 with reduced staining of LM-332 *(A)*. Additional mutation in intron 7, 628+42G→A, in keratinocytes of the biopsy of the lower leg with bright uninterrupted staining *(B)*. The cryptic splice site, CAG | GT, which is used when the 628+42G→A substitution was present, is indicated by the dashed line. The second-site mutation 596G→C was present in the keratinocytes with bright LM-332 staining isolated from the mosaic skin biopsy of the lower leg *(C)*. Red arrows point to the inherited mutation and green arrows to the second-site mutations. Corresponding amino acid sequences are indicated above the nucleotide sequences.

To determine the consequences of the 628G→A and the additional substitutions, 596G→C and 628+42G→A, on the mRNA splicing we isolated RNA directly from skin sections. cDNA was synthesized and analyzed by nested RT-PCR using exonic primers that amplified nucleotides 580-848 of *LAMB3* cDNA. In addition to the expected 269 fragment (fig. 5*A*, lane 1) the RNA isolated from affected skin revealed the presence of three aberrant mRNA transcripts (fig. 5*A*, lanes 2 and 3). Cloning and sequencing showed the correspondence of the 269 bp cDNA fragment to the full-length mRNA transcript (fig. 5*B*, transcript b). Furthermore, the band at approximately 200 bp consisted of two transcripts: (1) a transcript with exon 7 deleted (fig. 5*B*, transcript c), (2) a transcript with the deletion of exon 7 together with the first 2 bp (AG) of exon 8 (fig. 5*B*, transcript d). Exon 7 contains 64 bp and consequently skipping results in out-of-frame reading and most likely to nonsense-mediated RNA decay. The additional deletion of 2 bp of exon 8 in mRNA transcript leads to an in-frame skip, possibly giving rise to a smaller LM-332 β3-chain. The fourth mRNA transcript, the fastest migrating transcript, lacked exon 7 and the first 101 bp of exon 8; a deletion of 165 bp in total (fig. 5*B*, transcript e). This deletion of 165 bp is also in-frame and possibly renders a smaller β3-chain protein as well.
Interestingly, in the RNA isolated from the completely reverted biopsy specimen also a slower migrating amplimer on agarose gel was visualized, approximately 335 bp in size (fig. 5*A*, lane 5). Subsequent cloning of this product and sequencing showed this transcript to be in-frame with a retention of the first 66 bp of intron 7 (fig. 5*B*, transcript a). In previous work [23] RNA isolation of mosaic skin biopsy specimens was carried out by staining every fourth section and selecting on basis of the staining pattern of those sections. However, for a good selection on basis of other skin sections the reduced and bright interruptions along the epidermal-dermal junction should be of a sufficient length. Since in the mosaic biopsy of the lower leg the reduced areas and bright areas were interchanging over short distances -sometimes less than 10 basal cells- (fig. 2*D*), we chose to use RNA isolated from the whole skin biopsy, bearing in mind that about 50% of the cells were positive and 50% reduced for LM-332. Clearly visible is that RNA isolated from this biopsy showed a different distribution of mRNA transcripts (fig. 5*A*, lane 4) compared to biopsies taken from affected skin. The mRNA transcripts that result from alternative splicing due to the transition in the last nucleotide of exon 7 are less abundant (fig. 5*B*, transcripts c, d and e). Instead, almost all PCR product existed of the normal spliced transcript of 269 bp. Cloning of these normal-sized transcripts showed that a large proportion carried both the 596G→C and the 628G→A nucleotide changes. In concert with the absence of the 628+42G→A second-site mutation in intron 7 is the lack of the 335-bp amplimer.

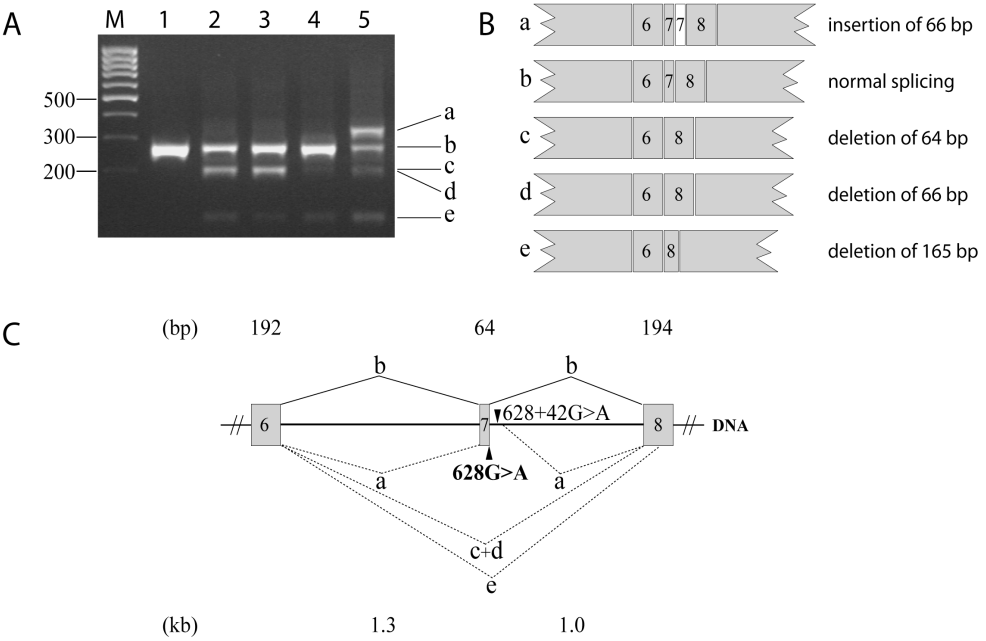

Figure 5 Effects of 628G→A and 628+42G→A at the mRNA level. The mRNA from skin sections was analyzed by RT-PCR using oligonucleotide primers that amplify nt 580-848 of LAMB3 cDNA (*A*). In addition to the expected 269 bp amplimer present in the control (lane 1), analysis of the amplification products of affected skin of the patient revealed two smaller transcripts (lanes 2-3). Both in RNA isolated from the lesional affected skin of the upper arm (lane 2) and RNA isolated from nonlesional affected skin of the upper arm (lane 3) transcripts of approximately 200 bp and 100 bp were observed. Isolation and sequencing of all amplimers showed the presence of the full-length normal-spliced transcript (*B*-*b*), the exon 7-deleted transcript (*B*-*c*), a transcript missing the first 2 bp of exon 8 in addition to exon 7 (*B*-*d*), and a transcript with a deletion of 165 bp, consisting of exon 7 and the first 101 bp of exon 8 (*B*-*e*). In the mosaic unaffected skin biopsy (lane 4) the normal-spliced variant was more abundant, whereas the amount of aberrant mRNA transcripts was decreased. Apart from the mRNAs present in keratinocytes from affected skin, a slower migrating product of 335 bp was observed in the completely reverted biopsy (lane 5). This mRNA transcript retained the first 66 bp of intron 7 (*B*-*a*). A 100-bp molecular size marker was used (lane M). A schematic representation of the genomic structure of the *LAMB3* gene extending from exon 6 to exon 8 (*C*), with the sizes of exons and introns given above and below respectively. The splicing of the normal full-length transcript is indicated with the solid line (b), whereas with the dashed lines the splicing of the aberrant transcripts is depicted (a, c, d and e). The mutations 628G→A and 628G+42G→A are marked by black arrows.

Nested RT-PCR was also performed with oligonucleotides amplifying bp 1641-2229 of *LAMB3* cDNA around the other inherited mutation 1903C→T. In keratinocytes of a healthy individual the expected 589-nt mRNA transcript was clearly visible (fig. 6, lane 1), whereas in all the biopsies of the patient, affected (fig. 6, lanes 2-3) and unaffected (fig. 6, lanes 4-5), this fragment was reduced in abundance. The other faster migrating transcript which comprised 210 bp lacked exon 14. As exon 14 is 379 bp, this mRNA transcript is out-of-frame and therefore prone to nonsense-mediated RNA decay. As the RT-PCR data around exon 14 of unaffected and affected skin were identical and no DNA changes were found, reversion of the inherited R635X mutation did not occur.

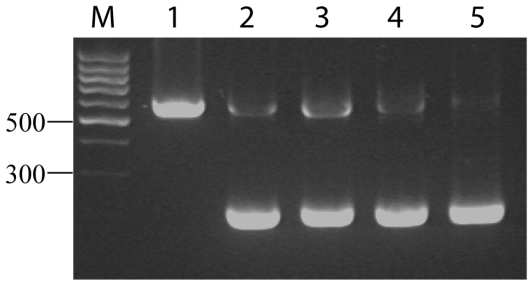

Figure 6 R635X induces exon 14 skipping. Exonic primers amplifying nt 1641-2229 of *LAMB3* cDNA showed the presence of the expected 589 transcript in the control (lane 1). This fragment was almost absent in biopsies of affected skin (lanes 2-3), mosaic unaffected skin (lane 4) as well as in completely reverted skin (lane 5). Interestingly, a smaller amplimer of 210 bp lacking exon 14 was visualized that was not present in the healthy individual's cDNA. A 100-bp molecular size marker was used (lane M).

Patient EB 029-01

The second GABEB proband, at present 63-years-old, has been described elsewhere [10,24]. He had the clinical characteristics of GABEB, with generalized blistering since birth of skin and mucosa, atrophic alopecia, nail dystrophy and dental anomalies. His consanguineous parents were unaffected, whereas his sister had the same disease. The grandparents were first cousins. IF of nonlesional affected skin specimens showed severely reduced LM-332 expression with monoclonal antibody GB3, whereas the type XVII collagen staining was indistinguishable from control skin (moAbs 1A8C and 1D1) [10]. In accordance, ultrastructural analysis showed hypoplastic hemidesmosomes in nonlesional affected skin [10]. Mutation screening identified the homozygous G→A transition affecting the last nucleotide of exon 7 (628G→A) [24].

Revertant patches on shoulder and upper arm

Because of the identification of multiple reversion events in the two *COL17A1* mosaic patients [23] and the previously described *LAMB3* patient, we started asking patients who consult our EB clinic if they have patches with clinically unaffected skin on a routine basis. Early 2006, patient EB 029-01 indicated three unaffected regions located on his shoulder and upper arm (fig. 7). Unfortunately, he could not remember how long these patches had been present and if they had expanded in time. Older photo-material was lacking. IF microscopy of the biopsy specimen taken from the unaffected revertant skin of the upper arm showed uninterrupted bright staining along the epidermal-dermal junction comparable with normal control skin (3+) (data not shown), confirming this EB patient as another individual with revertant mosaicism.

Figure 7 Presence of revertant unaffected skin on upper arm and shoulder of patient EB 029-01 in January 2006. The region where the revertant skin biopsy specimen was taken is circled, whereas other skin areas without blistering tendency are indicated with a cross *(A)*. A close-up from the revertant skin on the shoulder *(B)*. Both circle-form regions are clearly distinguishable from the surrounding reddish, erosive skin.

A third second-site mutation correcting 628G→A

Next, RNA was analyzed from skin sections of the revertant biopsy using nested PCR with primers amplifying nucleotides 580-848 from the LAMB3 cDNA. Since no normal spliced or larger amplimer was formed, the previously identified second-site mutations 596G→C and 628+42G→A could not be present in these revertant keratinocytes (fig. 8, lane 2). A true back reversion mechanism as well as the two second-site mutations found in patient EB 078-01 would have resulted in either a product of 269 or 335 bp. Subsequent DNA-analysis of the revertant keratinocytes by nested PCRs of exons 6, 7 and 8 using primers described in table 1, indeed revealed a different second-site mutation, 629-1G→A. This mutation is located in the acceptor splice site of intron 7 (fig. 9), and not present in the keratinocytes with reduced staining of the biopsy from nonlesional affected skin. Further analysis of RT-PCR products showed that the fastest migrating transcript had a deletion of exon 7 and the first 101 bp of exon 8 (fig. 5*B*, transcript e). Cloning did not reveal the out-of-frame 64-bp deleted transcript of exon 7 (fig. 5*B*, transcript c), however the in-frame 66-bp deleted transcript (fig. 5*B*, transcript d) was present in all selected clones with an insert size of approximately 200 bp. Apparently, the additional acceptor splice-site mutation results in the preferential use of the cryptic splice site 2 nucleotides downstream of the wild-type acceptor splice site of intron

7. This in-frame transcript with a 66-bp deletion results in a smaller LM-332 protein product and consequently in restoration of skin function.

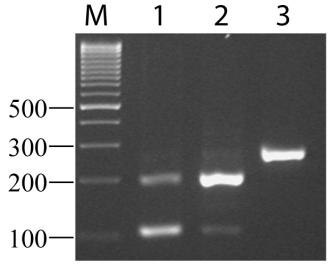

Figure 8 Effect of the second-site mutation 629-1G→A on mRNA. RT-PCR on cDNA obtained from skin sections of affected skin (lane 1) and revertant skin (lane 2) of patient EB 029-01 was performed amplifying nt 580-848 of LAMB3 cDNA. The control sample (lane 3) shows a transcript of 269 bp. Like expected, analysis of the amplification products of affected skin of the patient revealed, besides a low amount of the normal spliced transcript (fig. 5*B-b*), three smaller fragments due to the transition 628G→A. These smaller transcripts consist of the exon 7-deleted transcript (fig. 5*B-c*), a transcript missing the first 2 bp of exon 8 in addition to exon 7 (fig. 5*B-d*), and a transcript with a deletion of 165 bp, consisting of exon 7 and the first 101 bp of exon 8 (fig. 5*B-e*). In the skin biopsy of the revertant skin of the upper arm (lane 2) the distribution between the different aberrant transcripts has changed. The abundance of the smaller-deleted transcript(s) is higher. A 100-bp molecular size marker was used (lane M).

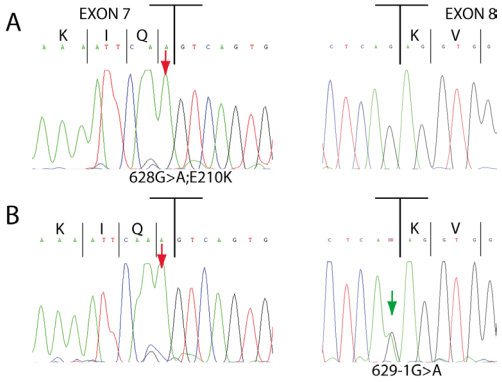

Figure 9 A new second-site mutation in the 3' splice site of intron 7. The inherited homozygous 628G→A mutation is present in both the proband's keratinocytes with reduced LM-332 staining (*A*) and the revertant keratinocytes (*B*). Additional mutation in the 3' splice site of intron 7, 629-1G→A, in keratinocytes of the biopsy specimen of the right upper arm (*B*). Red arrows point to the inherited mutation and green arrows to the second-site mutations. Amino acid sequences are indicated above the nucleotide sequences.

Discussion

This study in two patients with GABEB describes for the first time revertant mosaicism of LM-332 (fig. 10). The different second-site mutations present in the two biopsies of revertant skin of patient EB 078-01, make this patient another example of an individual with multiple reversion events [23,25,26]. Although we only identified one *in vivo* reversion in patient EB 029-10, it is well possible that in the two other revertant patches on his shoulder, indicated by crosses (fig. 7), other reversion

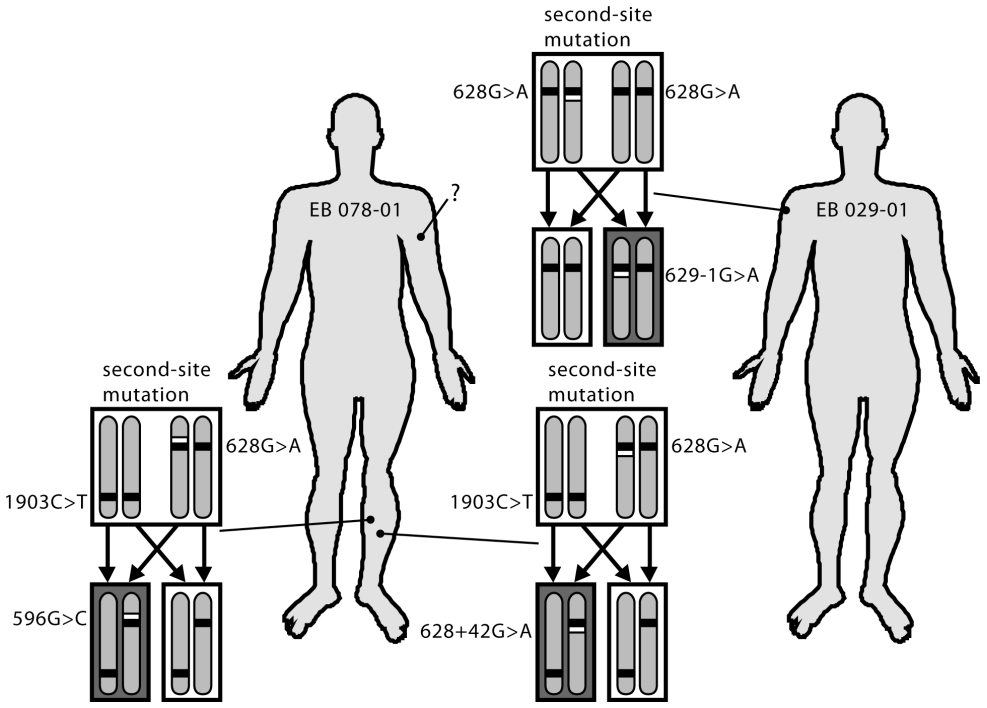

Figure 10 Schematic drawing indicating the different second-site mutations all correcting the same 628G→A mutation. In the revertant skin of the lower leg of patient EB 078-01 two different second-site mutations (white bars) were present. The schematic drawings indicate diploid cells after DNA replication and normal segregation. Homologous chromosomes carry the inherited mutations in the *LAMB3* gene (black bars). The cells with a mutant phenotype are in white, whereas the cells with a revertant phenotype are depicted in gray. Even more, the revertant cells in the upper arm specimen of patient EB 029-01 showed another second-site mutation. The *in vivo* reversion in the upper arm of proband EB 078-01 could not be identified due to the too small amount of revertant cells.

events took place. Last year different research groups separately identified the phenomenon of multiple reversion events within one patient, where before only one single mechanism was thought to occur in an individual patient. Multiple reversions were observed for different genes: the *FAH* gene in tyrosinaemia type I [25], the *RAG1* gene in Omenn Syndrome [26] and the *COL17A1* gene in EB [23]. Here, we add the *LAMB3* gene in EB.

All rescue mechanisms involved nucleotide changes in the DNA, a 596G→C change in exon 7 in the mosaic skin biopsy of proband EB 078-01, a G→A change 42 nucleotides from the exon/intron 7 border in the completely reverted skin biopsy of proband EB 078-01 and a G→A change in the 3' acceptor splice site of intron 7 in proband EB 029-01. The number of compensatory second-site mutations observed in inherited disorders is rapidly growing. Recently, in WAS a substitution in the initiation codon led to the use of a second ATG downstream of the inherited frame

shift-causing mutation, c.11delG; p.G4fsX40 [27]. These lymphocytes expressed WASP that lacked 5 N-terminal amino acids. Since the mutation occurred after chemo- or radiotherapies, it was hypothesized that these therapies might have elicited the mutation. Another suggestion for the occurrence of reversion mutations was proposed by Hamanoue et al [28]. The authors suggested that the Poly(T) sequence surrounding 2546C in the *FANCA* gene was likely to be susceptible to both forward and reverse mutations, explaining the recurrence of reversion at this site in different individuals. The *FANCA* gene is rich in direct repeats and homonucleotide tracts, which frequently cause small insertions and deletions partly through slipped-strand mispairing [29]. If such a region would be present in *LAMB3* in the neighborhood of the 628G→A mutation, the occurrence of the same second-site mutation would have been the most likely. As this was not the case, the cause of the correcting DNA events in our patients is yet unknown and may be the result of random mutagenesis.

Retrotransposon involvement

Recently, an alternative possibility of epigenetic revertant mosaicism caused by the action of a retrotransposon was proposed to explain mosaicism in autosomal recessive skin disorders [30]. A substantial part of the human genome, at least 40%, is derived from retroelements [31]. Retrotransposons, retroelements that replicate by transcription of an RNA intermediate, may move within the genome from one site to another, thereby affecting the activity of adjacent genes by methylation or demethylation, resulting in silencing or activation of gene expression respectively [32]. The establishment of the epigenetic state occurs during embryogenesis, i.e. the on/off switching of the e^{br} mutation in dogs gives rise to a brindled pattern following the lines of Blaschko [33]. Since silencing of a dominant negative allele may revert the phenotype but not that of a recessive allele, we propose that the retrotransposon proposition is more applicable to revertant mosaicism in autosomal dominant disorders, such as in EB simplex [22]. The retrotransposon hypothesis does also not explain the revertant phenotype in our patient, since we identified in the skin biopsy specimens of unaffected skin additional DNA changes in the *LAMB3* gene.

All second-site mutations affect mRNA splicing

The inherited G→A transition at the last base of exon 7 (628G→A) converts a negatively charged glutamic acid residue to a positively charged lysine (E210K). This substitution arises within the N-terminal globular (LN) domain of the short arm

of the LM-332 β3 chain, a region which has been postulated to be critical in the association of LM-332 with other structural proteins of the BMZ, including laminin-311 [34]. Thus this polarity change may result in a disturbance of the protein-protein interactions of LM-332 reducing the adhesion between the epidermis and the dermis. A more profound effect is however to be expected on the mRNA splicing since the 628 nucleotide change takes place in the consensus sequence at the 5' splice site. Effects of this common mutation in the *LAMB3* gene on the mRNA splicing have been described in several reports [9,24,35], of which Pulkkinen et al [24] investigated affected keratinocytes of patient EB 029-01. McGrath et al [9] reported on a GABEB family that carried besides the 628G→A also a 123C→T nonsense mutation in exon 3 [9]. In the family members no alternative mRNA transcripts were detected, even not by ASO (allele specific oligonucleotide hybridization) across the normal exon 6 – exon 7 borders and exon 7 – exon 8 borders. Although no splice variants were detected, the Northern blotting studies revealed a lower *LAMB3* signal (35% of control) than would be expected if mRNA splicing was normal from the E210K allele (about 50%). In fact, in another study by Posteraro and coworkers it was confirmed by amplification of nucleotides 353-672 of *LAMB3* cDNA that the mutation 628G→A caused aberrant mRNA splicing giving rise to two mRNAs [35]: one transcript is out-of-frame with the deletion of the 64 bp containing exon 7 and the second transcript originates from the activation of a cryptic splice site which uses the first two nucleotides (AG) of exon 8 as 3' acceptor splice site. This latter transcript is in-frame due to the deletion of the 64 bp of exon 7 and the first 2 bp of exon 8; it thereby codes for a β3 polypeptide lacking 22 amino acids within the LN domain of the short arm. Moreover, another study of patient EB 029-01 and his sister by Pulkkinen et al [24] showed that even more altered mRNA transcripts are formed due to the 628G→A mutation. By making use of oligonucleotide primers that amplified nucleotides 352-977 of *LAMB3* cDNA five distinct transcripts could be demonstrated. In addition to the transcripts found by Posteraro et al, two others were detected that derived from the use of cryptic splice sites within exon 6 and 8 [35]. Specifically, one mRNA transcript missing exon 7 and the first 101 bp of exon 8 and the other an in-frame deletion of 146 bp of exon 6 in addition to exon 7. The RT-PCR data of mRNA extracted from affected skin of patient EB 029-01 are slightly different from the data observed by Pulkkinen et al [24]. In contrast to their results, we observed a relatively lower abundance of normal-spliced transcript in the unaffected skin biopsy (fig. 8, lane 1). However, this is not that surprising, as Chavanas et al [36] already demonstrated that culturing conditions can influence splicing in the hemidesmosomal protein integrin α6β4. Pulkkinen et al [24] used mRNA from cultured keratinocytes, whereas we isolated mRNA directly from frozen

skin sections.

In both our patients three alternative mRNA transcripts were detected on agarose gel when using oligonucleotide primers that amplified nucleotides 580-848 of *LAMB3* cDNA (fig. 5*B*, transcripts c, d and e). More precisely: the out-of-frame exon 7-deleted (64 bp) variant, the in-frame transcript missing exon 7 and the first 2 bp of exon 8 (66 bp), and the in-frame transcript missing exon 7 and the first 101 bp of exon 8 (165 bp). Detection of the in-frame transcript missing 146 bp of exon 6 in addition to exon 7 described by Pulkkinen et al [24] was not feasible with the primers used.

Due to the correcting mutation 628+42G→A in patient EB 078-01, the cells of the completely reverted biopsy formed a larger mRNA transcript than normal with retention of the first 66 nucleotides of intron 7 due to the usage of a cryptic splice site in intron 7. This in-frame insertion will lead to the incorporation of a stretch of 22 amino acids –SQCGYFSCPWNYGWKRKQSWSP- in the LN domain of the β3 chain. Among the additional amino acids are two with basic side chains, four with acidic side chains, and six with bulky aromatic side chains. The 596G→C change results in an amino acid change of glycine to alanine at amino acid position 199, but more importantly effects mRNA splicing, giving rise to a higher production of normal-sized transcripts in the revertant cells.

RNA splicing is directed by the 5' donor splice site, 3' acceptor splice site and the branchpoint sequence, located 18-40 nucleotides upstream of the acceptor splice site (for review see [37]). In human these splicing sequences are poorly conserved; only the GT at the 5' end of the intron, AG at the 3' end of the intron and the branchpoint adenosine at the 2' position are almost invariably. Since natural splice sites can be different from the consensus sequence, both splice-site strength and accessory regulatory sequences influence splice-site selection. Different Web-based resources for splice-site prediction are available; among them is the 'Automated splice site analyses' located at https://splice.cmh.edu. Computational analyses of genomic sequences showed that the 628G→A mutation weakens the *Ri* (individual information content) of the natural donor site from 8.2 to 5.1 bits. Besides this 5' splice site, two other donor sites with a high *Ri* value are present in the analyzed sequence. These sites are TGG|GT, with an *Ri* of 4.3 bits, and CAG|GT, with an *Ri* of 3.2 bits, located 37 nt and 66 nt away from the natural exon/intron 7 border respectively. The second-site mutation 628+42G→A lowers the *Ri* value of the donor site TGG|GT drastically, from 4.3 to 0.8 bits, whereas the CAG|GT site remains unchanged at 3.2 bits. Additionally, the online computational method RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese) was used to look for possible exonic splicing enhancers (ESEs) that strongly affect spliceosome assembly [38]. ESEs

enhance splicing when present downstream of a 3' splice site and/or upstream of a 5' splice site. The program predicts that the 628+42G→A change gives rise to three new ESEs (TGGAAA, GGAAAA, GAAAAG), whereas no alterations occur of known ESEs. Thus, the fact that a cryptic splice site is recognized by the spliceosome instead of the natural splice site might be explained by the weakening of the TGG|GT donor site in combination with the creation of the new ESEs due to the 628+42G→A change.

For the 596G→C nucleotide change the *Ri* value of the natural donor site also remained 5.1 bits. The mutation, however, increased the *Ri* value of a cryptic acceptor site located in exon 7, from 2.5 to 4.1 bits. This cryptic acceptor site is not used, shown by the RT-PCR data. By using RESCUE-ESE it was excluded that this nucleotide change resulted in alterations of known ESEs. Another factor influencing splice-site selection which has not been considered is the possible effect of the 596G→C mutation on the RNA secondary structure neighboring the 628G→A mutation. Recently, Wessagowit et al [39] reported that the missense mutation 341G→T in the *COL7A1* gene, converting the amino acid glycine (GGG) to valine (GTG), resulted in the use of a new strong cryptic donor splice site. The alternative transcript carried an in-frame deletion of 29 amino acids in the NC-1 domain. Another exonic nucleotide change that effected splicing was described by Buchroithner et al [40]. The silent nucleotide change 3009C→T in exon 20 of the *LAMB3* gene, GGC as well as GGT both code for the amino acid glycine, creates a new exonic splice site AGGT, which competes with the natural donor splice site at the exon/intron 20 border [41].

Besides the wild-type 3' acceptor site of intron 7, CAG|AG, another cryptic splice site, GAG|GT, is present two nucleotides downstream from this wild-type acceptor site. Due to this cryptic splice site, 628G→A not only generates a 64-bp deleted transcript, but also the 66-bp deleted transcript. The compensatory mutation present in the revertant keratinocytes of patient EB 029-01, 629-1G→A, reduces the strength of the natural acceptor site of intron 7 from 10.5 to 6.0 bits, whereas the cryptic splice site increases in strength from 2.9 to 7.8 bits. RT-PCR analysis showed that the cryptic splice site is preferentially used and results in in-frame transcript instead of out-of-frame transcript, finally leading to a smaller LM-332 protein and skin without blistering tendency.

R635X mutation results in exon 14 skipping

The other inherited mutation, R635X, which is the most common mutation in junctional EB patients with a European origin, predicts a PTC within the coiled-coil

rod of the LM-332 protein. In a study of 14 families with the Herlitz junctional EB type, 64% of *LAMB3* affected alleles harbored the R635X mutation [42]. Although this mutation may result in translation of a truncated β3 chain polypeptide, it will probably result in a markedly reduced level of the corresponding transcript due to nonsense-mediated RNA decay, and consequently the absence of LM-332 from the skin. RT-PCR around exon 14 showed the reduced presence of the 589 amplimer, in line with the findings described for the patient homozygous for the R635X mutation as described by Pulkkinen et al [6]. In their case Northern blotting revealed the lack of *LAMB3* mRNA transcript. Surprisingly, in our patient on agarose gel also a smaller migrating transcript of 210 bp was visualized, belonging to an out-of-frame transcript with a deletion of exon 14 (379 bp). Although this exon 14-deleted transcript has not been reported before, it is not uncommon that PTCs induce exon skipping [41].

Implications for EB treatment

Our data have important implications for EB treatment. One of the major concerns in using gene or protein therapy is that the patient will develop an unwanted immune response to the formed novel protein. This introduced protein could be recognized as foreign, thereby tiggering an autoimmune response. Woodley et al [43] performed research on protein therapy by intradermally injecting human recombinant type VII collagen into mouse skin. Antibodies were made in 6 out of 10 mice. In case of all our mosaic EB patients with revertant mosaicism such a response did not occur. Patients with antibodies against type XVII collagen (bullous pemphigoid) or LM-332 (anti-epiligrin cicatricial pemphigoid) develop blisters. If such antibodies would be generated than skin fragility due to the autoimmune mediated blistering would be expected. Nevertheless this was not the case, probably because both patients still produced a small amount of deficient protein in their affected skin as shown by the reduced staining of LM-332. Similarly, the type XVII collagen mosaic patients described before also had residual staining with type XVII collagen-specific monoclonal antibodies 233 and NCC-Lu-226 [44].

In contrast to reverted skin of type XVII collagen mosaic patients the unaffected skin of the left lower leg of patient EB 078-01 expanded [23]. Previously, we suggested that expansion of clinically healthy skin area would be determined by the selection advantage of revertant stem cells compared to their deficient counterparts [23]. Inducible mouse models of EB simplex and epidermolytic hyperkeratosis with mutations in the keratin 14 and 10 gene, respectively, have shown growth advantage of stem cells expressing keratin 14, whereas stem cells expressing keratin 10 were

not favored above keratin 10-deficient stem cells [45,46]. It however is not clear if expressing LM-332 truly provides a growth advantage for the revertant stem cells, since although according to patient EB 078-01 his revertant skin patch increased in size patient EB 029-01 could not remember a clear expansion of his healthy skin area. It cannot be ruled out that the size of the β3-chain, which differs between these two patients is of influence here. LM-332 is involved in cell locomotion and migration as for example in wound healing [14,47]. Interactions with α2β1 and α3β1 are important for cell attachment, spreading and migration, whereas binding to α6β4 results in stable anchorage without cell spreading [48,49]. Suppression of endogeneous LM-332 in an oral squamous cell carcinoma cell line led to decreased cell attachment and increased migration [15]. It could be that the possible benefit of reversion is related with the role of LM-332 in migration, however this potential selection advantage of LM-332 revertant stem cells remains to be investigated.

Since the revertant patches remained stable in the type XVII collagen mosaic patients [23] and one healthy skin area involved two fingers of the female patient, we then concluded that in her case the reversion probably had taken place during embryogenesis. Although the healthy skin area expanded during life in patient EB 078-01, it still could be that the actual reversion mechanisms underlying the higher LM-332 expression occurred each in one cell early in embryogenesis that later in life expanded.

Revertant mosaicism in EB is believed to be unusual, although our recent observations indicate that it probably is not that uncommon. First, not only *COL17A1* and *KRT14* corrections have been identified in EB, but here we also show *in vivo* reversion of *LAMB3*. Second, we again observed revertant keratinocytes in affected skin, similar to patients described by Darling et al [20] and Pasmooij et al [23]. Third, at least two different reversion events took place in patient EB 078-01. The major benefit of this 'natural gene therapy' could lie in a cell therapy approach. Recently, Mavilio et al [50] have shown in a Phase-I clinical trial the correction of LM-332 deficient junctional EB by transduction of retroviral vector expressing β3 cDNA. A 30-yr-old male was transplanted with cultured epidermal sheets on both legs after removal of the outer skin layer. Transplantation was successful, revealed by the absence of blistering or inflammation for the duration of the follow-up, 4 months. In *LAMB3* revertant mosaic patients one might take advantage of the patient's own naturally corrected cells for skin transplantation. In such an approach there is no need to genetically modify epidermal stem cells.

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